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Isozyme uniformity in a wild extinct insular plant, *Lysimachia minoricensis* J.J. Rodr. (Primulaceae)

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Abstract

Isozyme analyses were conducted to evaluate the genetic diversity of seed accessions of *Lysimachia minoricensis* (a Balearic endemic plant that became extinct in the wild) provided by 10 European botanical gardens. No isozyme variation was detected after examination of the electrophoretic patterns of 22 putative loci in more than 150 plants. The lack of genetic variation in *L. minoricensis* is probably due to the shortage of sample propagules originally recovered in the field before its extinction. Extant plants of *L. minoricensis* are believed to have originated from a single source, therefore limiting their use as seed stocks for restoration projects.

Keywords: Balearic islands, *ex situ* conservation, germplasm accessions

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Introduction

Dramatic changes in demographical patterns and distribution areas can ultimately lead to extinction events of plant species through several unrelated stochastic processes affecting the survival of wild populations. Habitat disturbance, fluctuating environments, niche competition, pests, predation, introgression and hybridization with relatives, and extensive recollection for economic or museistic purposes are the main external factors responsible for plant extinction (Leigh 1981; Lande & Orzack 1988; Lande 1988; Gilpin 1990; Harrison 1991; Menges 1992; Nunney & Campbell 1993; Thomas 1994; Levin *et al*. 1996). Moreover, a lack of genetic variability to counteract unfavourable environmental changes has been considered the most decisive factor for the survival of plant species restricted to small areas and with low population size (Ellstrand & Elam 1993; O'Brien 1994). Therefore, one of the main goals in conservation biology is to assess levels of genetic variation, and how they are partitioned in endangered plant populations (Hamrick & Godt 1996).

Extinctions of vascular plant taxa from the Mediterranean area have been documented for over 30 taxa, excluding doubtful taxa and equivocal extinction events (Greuter 1994). Three of them, emphasizing the vulnerability of Mediterranean island floras, are single island endemics, *Diplotaxis siettiana* Maire (Alboran islet), *Dianthus multinervis* Vis. (Jabuka islet) and *Lysimachia minoricensis* J.J. Rodr. (Minorca island). According to conservation categories developed by the IUCN (1994), extinct taxa may be included in two categories, strictly extinct or extinct in the wild, depending on whether the organism has been preserved through any of the several *ex situ* conservation techniques available for plant conservation. Three of the 30 extinct Mediterranean taxa (*D. siettiana*, *L. minoricensis* and *Tulipa sprengeri* Baker) are still extant in cultivation and/or seed banks (Greuter 1994).

Plants extinct in the wild, but preserved alive in scientific institutions, provide suitable case studies to: (i) determine what levels of genetic variability have been preserved and how they are partitioned between germplasm accessions; and (ii) improve conservation strategies and recovery programmes by identifying genetically diverse germplasm resources.

We have addressed the study of the genetic variability of *L. minoricensis* (Primulaceae), a wild extinct endemic plant from the Balearic islands but widely distributed in European botanical gardens, using isozyme analysis. Despite its acknowledged limitations in contrast with powerful DNA-based applications, isozyme electrophoresis has been for a long time the favourite method to explore genetic variation in a wide taxonomic array of plants, both for taxonomic or conservation goals. The accumulated knowledge on plant isozyme variation of the past two decades has allowed useful standardization of

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data between taxa for comparative purposes (Brown 1978; Gottlieb 1981; Loveless & Hamrick 1984; Karron 1987; Hamrick & Godt 1989; Hamrick 1989; Hamrick *et al*. 1991).

L. minoricensis

L. minoricensis was described from a single population located at the south of the island of Minorca (Rodríguez 1869) and this was the only known site for the species until its extinction in the wild. At the beginning of this century, the plant was reported to be still alive (Rodríguez 1904) and no contrary statements were made by Knoche (1921). However, the field trips of Knoche to Minorca were made between 1908 and 1912 (Knoche 1921, p. 13). No other data were available until Bolós (1962) reported that seeds of wild *L. minoricensis* were gathered in 1926 and later planted at the botanical garden of Barcelona. It seems that the plant disappeared in the field between that date and 1950, as it was not reported by Montserrat (1953) who explored the original locality in that year . Since then the plant has been considered extinct in the wild (Sainz-Ollero & Hernández-Bermejo 1981; Gómez-Campo 1987) and recent explorations to the locality made by several teams of local botanists failed to find the plant (P. Fraga, personal communication). Plants growing at the botanical garden of Barcelona were thought to have disappeared as a consequence of disturbances during the Spanish civil war (1936–1939). However, the plant was later relocated in the garden and seeds were distributed to several European botanical gardens (Bolós 1962).

L. minoricensis was unsuccessfully re-introduced at the original site in 1959 (Bolós 1962) and 1989, but a new population was established in 1996 in another locality (Mayol 1997). Few biological features of *L. minoricensis* are known. The plant has a biennial cycle, although some individuals can develop flowering stems in the first year and others may survive the first flowering period and last several flowering seasons. The flowers are small (less than 5 mm), with green-brown petals. The mating system of the plant has not so far been studied, although autogamy is possible as isolated plants can develop viable seeds.

Materials and methods

Seeds of *Lysimachia minoricensis* were requested from the most outstanding European botanical gardens and a positive answer was obtained from 11 of them. Germplasm accessions from Kew Gardens were received, but after germination the plants showed little vigour and died at early stages of development. The origin of each accession was obtained whenever available and the resulting network is shown in Fig. 1. A total of 158 plants [Cambridge (20), Brest (20), Berlin (20), Paris (13), Belgium (18), Nancy (20), Córdoba (18), Sóller (8), Copenhagen (15), Edinburgh (6)] completed their vegetative growing successfully and were used for the isozyme analysis.

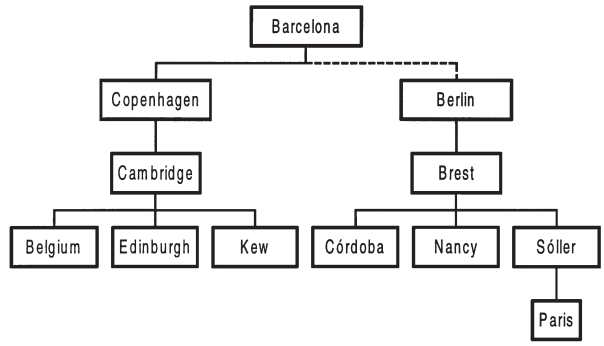

Fig. 1 Network depicting the source of the *Lysimachia minoricensis* accessions used in this work. The origin of the Berlin plants is unknown, but probably points to the Barcelona botanical garden.

Leaves were harvested and homogenized in the following extraction buffer, modified from Soltis *et al*. (1983): 0.1 N Tris-HCl pH 8.5, 6% (w/v) PVP-40, 0.02% (w/v) ethylenediaminetetraacetic acid (EDTA), 0.2% (w/v) $MgCl_2$, 0.36% (v/v) 2-mercaptoethanol added just before grinding to avoid oxidation, and 0.4% (w/v) bovine serum albumin (BSA). The extraction process consisted of crushing 300 mg of fresh leaves in a mortar and adding 0.9 mL of cold extraction buffer. The extracts were run in vertical discontinuous acrylamide gels (stacking gel at 4% in 0.125 M Tris-HCl, pH 6.8; separating gel at 7.5–10%, depending on the enzymatic system, in 0.375 M Tris-HCl, pH 8.8) with a constant voltage of 120 V for the stacking gel and 200 V for the separating gel. The gels were run until a day marker (bromophenol blue) was 10 cm from the origin.

Well-resolved bands were produced for 13 enzymes using the electrode buffers indicated in Table 1. Staining schedules followed Wendel & Weeden (1989), with the exception of those for esterase (Soltis *et al*. 1983) and for amylase (Murphy *et al*. 1996). Superoxide dismutase (SOD) was resolved as a negative tinction in all dehydrogenase staining procedures. Another 11 enzymes, aconitase (ACO), adenylate kinase (ADK), endopeptidase (ENP), formate dehydrogenase (FDH), β-glucosidase (GLU), hexoquinase (HEX), isocitrate dehydrogenase (IDH), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), mannose phosphate isomerase (MPI), leucine aminopeptidase (LAP) and phosphoglucomutase (PGM), were not adequately resolved on the gel and electrode buffer systems assayed and so were not included in the study.

Results and Discussion

No electrophoretic variation was detected in more than 150 *Lysimachia minoricensis* plants with the experimental procedures used, as all plants showed the same phenotype

Table 1 Electrode buffers and enzyme resolution for the 27 enzyme systems assayed

	Buffers					
Enzyme	A	B	C	D	E	F
AAT	1	1	2	–	2	–
ACO	–	–	0	–	0	0
ACP	–	–	–	–	1	–
ADH	1	0	2	0	1	–
ADK	0	–	–	–	0	–
AMY	0	0	2	0	1	–
CAT	0	2	2	0	1	–
EST	1	0	1	–	2	–
F-EST	1	–	2	–	2	–
ENP	–	–	0	–	0	–
FDH	0	0	0	0	0	0
GDH	1	–	2	–	–	–
β-GLU	–	–	0	–	–	–
G6PD	1	1	2	–	2	–
HEX	–	0	0	0	0	–
IDH	0	0	–	0	–	0
LAP	0	0	0	0	0	–
LDH	0	1	1	0	1	–
ME	2	0	–	0	–	–
MDH	–	–	0	–	0	0
MPI	–	–	0	–	0	–
PGD	1	–	–	–	2	–
PGI	1	1	2	–	–	–
PGM	0	1	1	1	1	–
RBC	2	–	2	–	–	–
SOD	1	1	2	1	2	–
SKD	1	1	1	–	–	1

Buffer composition (anode/cathode): A, Tris-glycine (0.025 M Tris, 0.2 M glycine) pH 8.3/Tris-glycine (0.025 M Tris, 0.2 M glycine) pH 8.3; B, Tris-acetate (0.1 M) pH 8.9/Tris-glycine (0.025 M Tris, 0.2 M glycine) pH 8.3; C, Tris-glycine (0.025 M Tris, 0.2 M glycine) pH 8.3/Tris-acetate (0.1 M) pH 8.9; D, Tris-acetate (0.1 M) pH 8.9/Tris-alanine (0.08 M alanine, 0.01 M Tris) pH 8.9; E, Tris-alanine (0.08 M alanine, 0.01 M Tris) pH 8.9/Tris-acetate (0.1 M) pH 8.9; F, lithium-borate (0.03 M lithium hydroxide, 0.2 M boric acid) pH 8.3/lithium-borate (0.03 M lithium hydroxide, 0.2 M boric acid) pH 8.3. –, not tested; 0, no activity; 1, enzyme activity present, but poor resolution; 2, scorable bands.

patterns for all enzymes assayed. A conservative genetic interpretation of the monomorphic zymograms (Fig. 2) suggests that the number of presumptive loci encoding the 13 enzymes screened is 22. This was estimated from the known quaternary structure of the enzymes, the minimal conserved number of isozymes found in plants (Weeden & Wendel 1989), and the diploid nature of the species ($2n = 24$; Cardona & Contandriopoulos 1980). The electrophoretic pattern of GDH points to a gene duplication with two loci. To our knowledge this is the lowest genetic diversity found within Primulaceae.

On a theoretical basis, cryptic variability in *Lysimachia* plants could have been underscored due to limitations of the electrophoretic technique and the sample size used. Electrophoretic techniques are capable of resolving only a portion of the genetic variation, as: (i) only amino acid changes resulting in significant charge are easily detectable under the experimental conditions used; and (ii) a single electrophoretically determined mobility class may contain more than one allele. Although sequential electrophoresis may reveal additional electromorphs, it has been suggested that whereas polymorphic loci under any single experimental condition may be more variable under new electrophoretic setups, monomorphic loci sometimes remain largely invariant (Coyne 1982; Barbadilla *et al*. 1996). Low sample size can be responsible for some studies reporting a lack of genetic variability (Sjögren & Wyöni 1994). However, from the 158 sampled plants of *L. minoricensis* we should have a probability of 95.6% of detecting any variant allele that existed at least at an overall frequency of 1%.

To what extent is the lack of detectable genetic variation in *L. minoricensis* plants unexpected? Leaving aside plants having predominantly asexual, apomictic or vegetative reproduction (Ellstrand & Roose 1987), relatively few taxa (at least 45 taxa; cf. Godt *et al*. 1997) have been reported to lack detectable genetic variability using isozymic markers. Nevertheless, all these records refer to wild-sampled plants and no references are available concerning the genetic variation present in *ex situ* collections of wild extinct plants. Therefore, it should be assumed that our results do not strictly address the question of how much genetic variation was present in *L. minoricensis* before extinction, but rather what level of genetic variation, inferred from allozyme markers, has been preserved from extinction.

With the exception of the Berlin accession, whose origin could not be traced, all other *L. minoricensis* samples did originate, ultimately, from seeds supplied by the Barcelona botanical garden. The Berlin accession was obtained before 1974, but there is no sound reason to support that it was acquired from field plants before *Lysimachia* extinction. Hence, present evidence supports the view that all *ex situ* available collections of *L. minoricensis* kept in conservation institutions derived from a single source. Therefore, the genetic depauperation exhibited by extant *L. minoricensis* plants could also have been favoured by the exchange system among botanical gardens, a prominent problem not sufficiently appraised in plant conservation literature (but see Hurka (1994)).

The role of botanical gardens in plant conservation, as preservation of taxa from extinction through *ex situ* collections, could not be as straightforward and effective if genetic variation within species is underrepresented in their plant collections. *L. minoricensis* could serve as a case study to illustrate how often the preservation of species does not capture enough genetic variability for effective

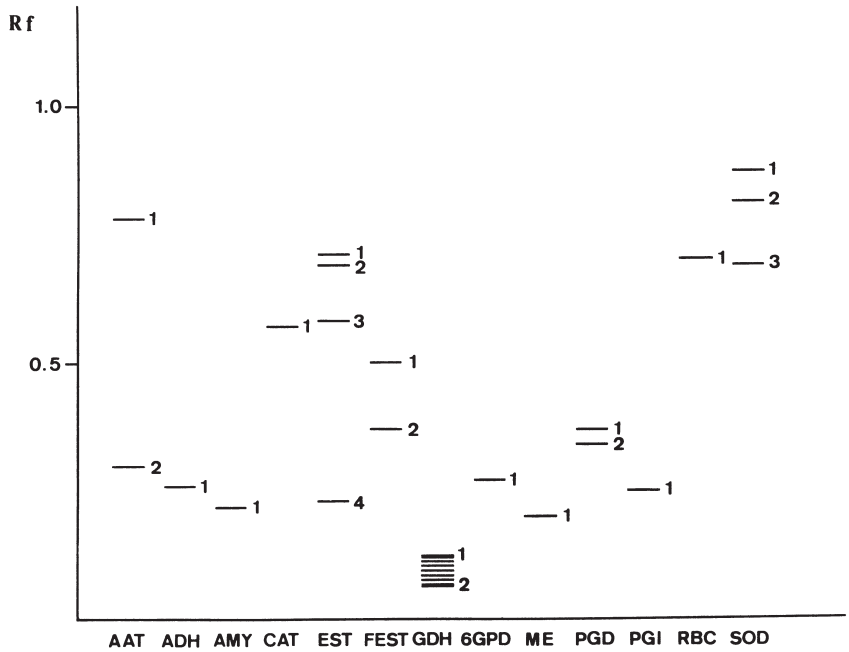

Fig. 2 Phenotypes of the 13 isozyme systems resolved in *Lysimachia minoricensis*. The numbers refer to putative isozyme loci.

conservation purposes. This could be a serious problem for many endangered endemic taxa inhabiting the Balearic islands (some of them only known from as few as one or two localities, e.g. *Apium bermejoi* L. Llorens, *Arenaria grandiflora* L. ssp. *bolosii* (Cañig.) P. Küpfer, *Brimeura duvigneaudii* (L. Llorens) Rosselló *et al*., *Cymbalaria fragilis* (J.J. Rodr.) A. Cheval, *Dianthus rupicola* Biv. ssp. *bocchoriana* L. Llorens & Gradaille, *Euphorbia fontqueriana* Greuter, *Genista dorycnifolia* Font Quer ssp. *grosii* (Font Quer) Font Quer & Rothm., *Ligusticum huteri* Porta, *Euphorbia margalidiana* Kuhbier & Lewej., *Ranunculus weylerii* Marès ex Willk., *Thymus herba-barona* Loisel. ssp. *bivalens* Mayol *et al*., *Vicia bifoliolata* J.J. Rodr.) which are prone to extinction in the wild in the short term. Some of these are preserved in several Spanish germplasm collections, but the lack of data about how much genetic diversity has been preserved adds a point of caution over the *ex situ* conservation measures taken so far.

Allozymes have long been the standard method for analysing population variability and differentiation in plants. However, their limitations for obtaining an accurate picture of the genetic variation of the whole genome has prompted the use of complementary molecular markers in conservation biology. Therefore, the use of additional more powerful tools for the estimation of the level of genetic variation has been applied in endangered organisms (Brauner *et al*. 1992; Gray 1995; Swensen *et al*. 1995; Palacios & González-Candelas 1997a,b). DNA fingerprinting methods could reveal whether the lack of genetic variation found in *L. minoricensis* merely reflects the pitfalls of the technique (one-dimensional electrophoresis) and the markers (allozymes) used or mirrors the genetic depauperation of the species. This should assist conservation managers in choosing the most genetically appropriate seed accession for restoration projects, a fact overlooked in previous recovery plans.

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